Current genetic engineering strategies for the production of anti-hypertensive ACEI peptides

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Abstract

Hypertension is a major risk factor for cardiovascular diseases, with high prevalence in low- and high-income countries. Among the various antihypertensive therapeutic strategies, synthetic Angiotensin I-converting enzyme inhibitors (ACEI) are one of the most used pharmacological agents. However, their use in hypertension therapy has been linked to various side effects. In recent years considerable research has been performed on the use of food-derived ACEI peptides (ACEIp) as antihypertensive agents. Although promising, the industrial production of these ACEIp through conventional methods, such as chemical synthesis and enzymatic hydrolysis of food proteins, has been proven troublesome and expensive. Limitations to the large-scale production of ACEIp for functional foods and supplements can be overcome by producing the precursors of these peptides in heterologous hosts. Bacterial hosts have been the privileged choice, particularly to test the success of the genetic engineering strategies, but new platforms based on plants and microalgae have also been emerging. This work provides an overview of the state of antihypertensive therapy, focusing on ACEI, illustrates the latest advances on ACEIp research, and describes current genetic engineer-based approaches for the heterologous production of ACEIp for antihypertensive therapy.

Hypertension and anti-hypertensive therapy

Hypertension – an overview

Hypertension (HT) is defined as a systolic blood pressure (BP) of [?]140 mmHg and/or a diastolic BP of [?]90 mmHg in young, middle-aged and elderly subjects (Williams et al., 2018). In 2004, HT was declared by the World Health Organization (WHO) as the lead risk factor for death worldwide, accounting for 7.5 million deaths (12.8% of total deaths). Hypertension is the leading risk factor for global disease burden (Lim et al., 2012) , particularly cardiovascular diseases, and an important risk factor for other diseases such as kidney failure. According to the Global Health Observatory data repository 2015**,** HT affects 1.13 billion people, with a global age-standardized prevalence of 22.1% (24.1% for males). In low-income countries, the high prevalence (28.4%) of hypertension is mainly attributed to flawed health systems, resulting in untreated and uncontrolled patients (Yeates et al., 2015). In high-income countries, hypertension prevalence levels are slightly decreased probably in response to public health actions (Lloyd-Jones and Levy, 2013; World Health Organization, 2013), however HT prevalence is expected to increase globally in coming years, due to the growth and ageing of world's population, behavioral risk factors and lack of efficient therapies.

Hypertension is classified as primary or secondary according to its underlying cause. Most HT patients (˜95%) have primary HT, which is defined as high BP without secondary causes. The remaining 5% have secondary HT, deriving from a known medical condition ('secondary cause') that is often reversible, (e.g. obstructive sleep apnea, renovascular disease, renal failure) (Carretero and Oparil, 2000; Oparil et al., 2003; Pullalarevu et al., 2014; Rimoldi et al., 2014). Conversely, primary HT has no single causative agent - it seems to be influenced by the presence of risk alleles and multiple other factors such as body mass index, gender, insulin resistance, high alcohol and salt intakes, low potassium and calcium intakes, stress, aging and sedentary lifestyle (Carretero and Oparil, 2000; Pullalarevu et al., 2014). Although still scarcely identified, genetic factors are thought to play a major role in primary HT, and primary HT tends to be associated with parental HT (Wang et al., 2008) - family studies estimated the heritability of BP from 30 to 50% (Ehret and Caulfield, 2013). However, this complex polygenic disorder seems to be influenced by the interaction of genomic and environmental factors, challenging the clear identification of susceptibility genes and epigenetically modulated mechanisms. The advent of high-throughput genotyping technologies provided some clues on the contribution of common genetic variation on BP traits and primary HT (Ehret et al., 2016; Burrello et al., 2017; Warren et al., 2017), although additional studies are needed to confirm associations. Further, epigenetic marks (e.g. DNA methylation, histone modifications and non-coding RNAs) might provide missing links in BP traits variability (Richard et al., 2017).

Antihypertensive therapy

The central role of HT in the pathophysiology of many age-related chronic diseases of high prevalence and socioeconomic burden, boosts research on effective antihypertensive therapies. Their main goal is to lower HT-associated complications, morbidity and mortality (Berryman, 2000; Kamath, 1990), related to target-organ damage such as cardiovascular events, heart failure and kidney disease (Berryman, 2000). Controlled BP is the most feasible clinical end point, but patients should be guided on both pharmacological therapy and lifestyle modifications ('nonpharmacological therapy'), since BP reduction alone may not guarantee prevention of future target-organ damage (Wells et al., 2009; Koda-Kimble and Alldredge, 2013;). There are many factors to consider when choosing the right antihypertensive therapy, such as: concomitant illnesses and medications, HT stage, age, compliance, genetics and special populations (Wells et al., 2009; Clark et al., 2011; Koda-Kimble and Alldredge, 2013). Nonpharmacological therapy can include a combination of weight loss, restricted sodium intake, increased aerobic exercise, moderation in alcohol consumption, and stress relief (Brunton et al., 2005; Kasper et al., 2015; Gouveia et al., 2017). Nonpharmacological therapy may suffice in cases of prehypertension and, when combined with pharmacological regimens, can increase the drug therapy efficacy, improving the quality of life and longevity (Gouveia et al., 2017).

A great variety of pharmacological antihypertensive agents can effectively lower BP. Current *European Society of Cardiology and European Society of Hypertension* (ESC/ESH) guidelines an initial therapy with a combination of ACEI or angiotensin receptor blockers (ARBs) with diuretics (including thiazides, chlorthalidone and indapamide) or calcium channel blockers (CCB). A triple combination could be prescribed but concomitant use of ACEi and ARB is discouraged (Williams et al., 2018). As add-on therapy, alternative potent antihypertensive drugs (e.g. spironolactone) can additionally lower BP in patients with resistant hypertension although they have an high incidence of adverse effects (Berryman, 2000). Supplementary Table S1 summarizes some of the first line antihypertensive agents according to their subclasses, action mechanism, produced effects and clinical applications. A list of antihypertensive drugs and common therapies is also provided.

Angiotensin I-Converting Enzyme Inhibitors

Angiotensin I- Converting Enzyme (ACE) is a main target in hypertension therapy given its key role in the Renin-Angiotensin system, the main metabolic pathway regulating human BP and fluid homeostasis. The action of ACE increases BP as a result of both increased vasoconstriction and diminished vasodilation.

In the past twenty years, synthetic ACEI have been one of the preferred first-line therapy for HT, especially in diabetic and chronic kidney disease patients (TH et al., 2003; Jimsheena and Gowda, 2010; Zisaki et al., 2015; Perico et al., 2017). ACEI act on both the Renin-Angiotensin and the Kallikrein-Kinin systems (Figure 1). By blocking ACE activity, ACEI prevent the conversion of angiotensin I into the potent vasoconstrictor angiotensin II (Berryman, 2000). ACEI also block the breakdown, by ACE, of the vasodilatory peptide bradykinin, responsible for increasing the production at the blood vessels of two potent vasodilators: prostacyclin and nitric oxide (Clark et al., 2011). By reducing angiotensin II levels, ACEI also decrease the secretion of aldosterone, and thus sodium and water retention (Berryman, 2000). Overall, ACE inhibition decreases BP as a result of both increased vasodilation (due to the action of bradykinin at blood vessels) and diminished vasoconstriction (resulting from the lack of angiotensin II and its target aldosterone) (Figure 1) (Clark et al., 2011).

ACE inhibitors have been divided in three categories: 1) captopril; 2) prodrugs, such as enalapril and fosinopril; and 3) lisinopril, a water-soluble and the only non-metabolized ACEI (Zisaki et al., 2014). Most of ACEI are prodrugs, requiring hepatic conversion to pharmacologically active metabolites. Captopril and lisinopril are exceptions to this rule and are prescribed to patients with severe hepatic impairment (Kelly and O'Malley, 1990; Zisaki et al., 2014). As the majority of ACEI are eliminated primarily by the kidney, they may require dose adjustments to varying degrees of renal impairment (Weber, 1991; Piepho, 2000).

Although widely used for treating HT, congestive heart failure, and diabetic neuropathy (Jimsheena and Gowda, 2010; Zisaki et al., 2014) synthetic ACEI have been associated with various side effects including cough, skin rashes, hypotension, loss of taste, angioedema, reduced renal function and fetal abnormalities (Norris and FitzGerald, 2013). To minimize the risk of side effects, synthetic ACEI have been combined with other antihypertensive agents, such as calcium-channels blockers (Egan, 2007) (Supplementary Table S1). Moreover, naturally occurring ACEIp have gathered attention as potential antihypertensive agents to be used alone or in combination with other non-pharmacological therapies, for HT preventive measures and initial treatment of HT (Gouveia et al., 2017).

ACEI peptides research

Food-derived ACEIp

ACEI peptides derived from proteins of food sources have attracted great attention as antihypertensive agents. The major difference between these ACEIp and synthetic ACEI is that the first do not cause significant BP lowering effects on normotensive subjects, avoiding acute hypotensive effects. Emerging evidence suggests that these food-derived peptides can act through other mechanisms besides ACE inhibition, such as the upregulation of ACE2 (an ACE homologue that counterbalances the detrimental effect of elevated ACE), endothelial function improvement, and reduced vascular oxidation and inflammation (Wu et al., 2017). Based on these findings, ACEIp have been proposed for the initial treatment of mildly hypertensive patients or as complementary treatment in hypertensive patients (Rosales-Mendoza et al., 2013).

Food-derived ACEIp have great potential as food additives/ingredients for novel functional foods/nutraceuticals, and as pharmaceutical ingredients for novel drug formulations. ACEIp have been identified in enzymatic hydrolysates of different food proteins, such as milk (Seppo et al., 2003; Miguel et al., 2006; Ruiz-Gimenez et al., 2012), egg (Asoodeh et al., 2012; Duan et al., 2014), plants (Marczak et al., 2006; Jakubczyk and Baraniak, 2014; Orona-Tamayo et al., 2015; Rayaprolu et al., 2015; Vásquez-Villanueva et al., 2015; Wu et al., 2016), meat (Castellano et al., 2013), fungi (Tran et al., 2014; Geng et al., 2015), and marine source proteins (Fujita and Yoshikawa, 1999; Balti et al., 2015; Ghanbari et al., 2015), and currently constitute the most well-known class of bioactive peptides. ACEIp can be enzymatically released from their precursor proteins during food processing and gastrointestinal digestion. An extensive overview of ACEIp isolated from food sources is given in Supplementary Table S2, while examples of commercially available nutraceuticals or food ingredients containing ACEIp are presented in Table 1. These nutraceuticals

are normally formulated in the form of beverages, capsules or powders that can be directly consumed or used as ingredients for further food or pharmaceutical applications. The main food sources of ACEIp are fermented milk, bonito and other traditional foods with empirical health benefits, consumed by humans long before the concepts of 'functional food' and 'bioactivity' even existed. Hence, the most well-known natural ACEIp-containing functional foods are fermented milk-derived, including milk-derived beverages and sour milk tablets (Table 1). ACEIp from marine sources, such as bonito, sardine and seaweeds, are also present in food ingredients and functional foods in a broad variety of formulations, including BP-lowering capsules and tablets (Table 1). These ACEIp-containing products can be naturally obtained through fermentation by specific microorganisms, or artificially through*in vitro* hydrolysis with gastrointestinal and commercial proteases (Hartmann and Meisel, 2007; Pihlanto and Mäkinen, 2013; Hayes and Tiwari, 2015)

Molecular determinants of ACEI peptides

The discovery of novel ACEIp was traditionally based on the analysis of whole food protein hydrolysates through wet chemistry techniques. However, this workflow has since significantly evolved and *in silico* methods play an increasingly important role in ACEIp research.*In silico* methods can be coupled to *in vitro* techniques in the screening and characterization of novel ACEIp (Sun et al., 2017). Particularly, the analysis of their ACE inhibitory activity based on the peptide primary structure, termed quantitative structure–activity relationship, has been widely used (Wu et al., 2006).

Considering results from analytical and chemometric studies, some rules were established concerning the primary structure of ACEIp. Potent ACEIp are generally short chain peptides (2-12 amino acids in length), although some larger inhibitory peptides were identified in fertilized egg (Duan et al., 2014), milk fermented with *Enterococcus faecalis* or the *Lactobacillus casei* strain Shirota (Quiros et al., 2007; Rojas-Ronquillo et al., 2012), koumiss (Chen et al., 2010), tuna (Lee et al., 2010), bonito (Hasan et al., 2006) and rotifer (Lee et al., 2009). Chain composition and amino acid position of ACEIp also play a role on the ACE inhibition potential. The N-terminal of potent ACEIp generally contains hydrophobic amino acids, especially those with aliphatic chains such as Gly, Ile, Leu, and Val (Iwaniak et al., 2014). The C-terminal tripeptide sequence of ACEIp (and ACE substrates) strongly influences ACEI activity, since ACE cleaves the C-terminal dipeptide of oligopeptide substrates with a wide specificity. Hydrophobic amino acid residues with aromatic or branched side chains at each of the C-terminal tripeptide positions are common features among potent inhibitors (Soares de Castro and Sato, 2015). In general, peptides showing higher activity against ACE have Pro, Tyr, Phe or Trp at their C-terminus (Norris and FitzGerald, 2013). Indeed, many potent food-derived ACEIp contain Pro residues at one or more positions in the C-terminal tripeptide region (Iwaniak et al., 2014). This rule concerns most particularly short-length peptides. Even though a similar pattern has been observed for long chain milk-derived peptides, their activity was generally not influenced by C-terminal Pro (Aluko, 2015),

The relationship between the peptides secondary structure and ACEI activity has also been analyzed (Yu et al., 2011). The elucidation of the crystal structure of a complex between human ACE and an inhibitor (Natesh et al., 2003) has provided a platform for analyzing ACEIp inhibitory mechanisms by molecular docking and molecular dynamics (MD) simulation (Xie et al., 2015). Molecular docking, a fast technique, has been coupled with accurate but time-consuming MD techniques in the discovery of novel inhibitory peptides and their mechanism of interaction with ACE at the molecular level (Alonso et al., 2006).

Finally, online *in silico* tools as ToxinPred and PeptideCutter can also be applied to predict the toxicity and enzymatic digestion of novel ACEIp (Sun et al., 2017).

In vitro and physiological ACE inhibitory activity

Following predictions by *in silico* techniques, the ACE inhibitory activity of novel ACEIp needs to be confirmed. *In vitro*inhibitory activity assays generally rely on spectrophotometric, fluorometric, colorimetric and radiochemical methods, as well as chromatography techniques (Murray and FitzGerald, 2007). The

measure of activity is usually given as the IC50, defined as the concentration of peptide required to inhibit ACE activity by 50% (Donkor et al., 2005). However, *in vitro* ACE inhibitory activity does not always conduce to a BP lowering effect, and ACE inhibitory activity assays must be performed *in vivo* . These assays generally consist in measuring the BP response in spontaneously hypertensive rats (SHR), following intravenous or intraperitoneal injection, or oral administration, of ACEIp or related extracts.

Data from the ACE *in vitro* inhibition and the *in vivo*BP-lowering effect have provided a basis to classify ACEIp into 3 categories (Ryan et al., 2011): 1) Inhibitor-type ACEI peptides resistant to cleavage by ACE, hence their activity is not significantly altered by binding to ACE; 2) Substrate-type ACEI peptides, showing a decrease in ACEI activity due to their cleavage by ACE upon binding; 3) Prodrug-type ACEI peptides, larger peptides that are converted to potent ACE inhibitors following hydrolysis by ACE or gastrointestinal proteases.

The susceptibility to cellular peptidases is another important factor determining the physiological activity of ACEIp. Some peptides may be deactivated by their susceptibility to degradation by gastrointestinal, intestinal and kidney brush borders, serum and blood proteinases and peptidases during transport to the target organ(s). Conversely, oligopeptide sequences containing encrypted ACEIp may be activated and their ACEI activity increased, following *in vivo* proteinase and peptidase activities (Tai et al., 2018). The intestinal absorption and bioavailability of peptidic ACEI fragments is also of paramount importance. ACEI should be able to cross the intestinal wall and sequentially enter the blood circulation in order to exert an antihypertensive effect. The Caco-2 cell (a human epithelial colorectal adenocarcinoma cell line) has been generally used as a model to investigate the stability of ACEIp during absorption/transepithelial transport. Usually, di-/tripeptides are absorbed across the brush border membrane in their intact forms, via specific peptide transporter systems, while others can be prone to protease degradation (Shen and Matsui, 2017).

Formulation/delivery strategies

During storage and/or food processing, the ACEIp activity may decrease due to partial degradation or destabilization by proteases, extreme conditions (e.g. of pH, temperature, oxygen) and/or by unwanted interaction with other components (cations, lipids, proteins, etc.). Protective strategies have been employed to maintain the bioavailability and antihypertensive activity of ACEIp directly included in food/nutraceuticals. Encapsulation solutions aiming at maintaining peptide activity during their shelf life or even upon consumption include the use of liposomes, chitosan particles, among others.

Encapsulation in food-grade liposomes, which can retain both hydrophilic and hydrophobic peptides, can protect and help the transport of bioactive peptides (Mozafari et al., 2008; Malheiros et al., 2010). Liposomes may even increase nutritional value if derived e.g. from natural soy lecithin or partially purified phosphatidylcholine, with high content in polyunsaturated fatty acid and low tocopherol content (Taladrid et al., 2017). Peptide-loaded liposomes may be easily introduced into functional foods via a film matrix, which helps avoiding unwanted strong flavors or peptide interactions with other constituents (da Silva Malheiros et al., 2010). Encapsulation of ACEIp into food grade chitosan nanoparticles (CNP) ensures safe oral administration and decreases gastrointestinal enzymatic degradation. Indeed, ACEIp stabilized by CNP were shown to more efficiently reduce BP for extended time periods in SHR (Auwal et al., 2017). Other encapsulation methods may also improve the bioavailability and efficacy of the ACEIp (Huang et al., 2017).

Finally, additives such as skim milk, sugars and sugar alcohols, soybean (*Glycine max*) casein, casein hydrolysate, and others help to mask the ACEIp's bitterness, and improve their applicability in functional foods/nutraceuticals (Iwaniak et al., 2016a; Iwaniak et al., 2016b; Pooja et al., 2017).

Genetic engineering ACEI peptides production

Large-scale production of ACEIp for functional foods and food supplements is still a challenge. Industrial production of ACEIp has been mainly based on enzymatic proteolysis of whole food proteins to release peptides with ACE inhibitory activity (Pihlanto and Mäkinen, 2013). However, such methods generate a complex mixture of compounds and lead to challenging target peptide isolation processes (Losacco et al., 2007). The high cost, low recovery and bioavailability of ACEIp produced by such current methods pushed the need to develop alternative approaches, as the production of ACEIp via heterologous expression platforms. Most recombinant ACEIp have been produced in*Escherichia coli,* the most widely studied heterologous expression host. Emerging platforms for ACEIp production include lactic acid bacteria (LAB), plants (predominantly cereals and legumes) and the microalgae *Chlamydomonas reinhardtii* .

The main bottleneck in the heterologous expression of short peptide chains as ACEIp is their susceptibility to proteolytic degradation inside the host system. Accordingly, direct expression of genetically engineered ACEIp has been proven troublesome. This section describes strategies to tackle this problem (design of efficient expression constructs, e.g. fusion proteins, tandem ACEIp; protein targeting approaches; and use of protease-deficient hosts such as *E. coli*BL21 (Rao et al., 2009)) and demonstrates the potential of heterologous expression systems to produce ACEIp at large-scale, low-cost and in convenient formulations.

Recombinant ACEI peptide expression in bacteria

Bacteria are well-established expression systems for high-level production of recombinant proteins and peptides, and have been the preferred systems for heterologous ACEIp expression (Losacco et al., 2007). *E. coli* is particularly used in new ACEIp production systems, and the use of LAB is an emerging alternative, given their Generally Recognized as Safe (GRAS) status.

Four main strategies have been adopted for ACEIp production in bacterial hosts: 1) generation of multimeric polypeptides containing tandem repeats of an ACEIp; 2) fusion of ACEIp to proteins; 3) generation of bioactive polypeptides containing multivariate ACEIp; and 4) mutation of plant storage proteins to include ACEIp. In all these approaches, the aim is to protect the ACEIp against degradation, as polypeptides and fusion proteins are less susceptible to degradation by host peptidases than single ACEIp. Table 2 presents several recombinant ACEIp produced in bacterial platforms, their production strategy, yield and bioactivity.

Tandem repeats of ACEIp

The expression of chimeric configurations containing tandem repeats of a desired ACEIp, flanked by protease recognition sequences that allow the peptide release after *in vitro* protease cleavage or gastrointestinal digestion showed significant advantages at the expression level (Kim et al., 2008).

One of the early examples of the use of ACEIp in tandem consisted of ten amino acids derived from the yeast GAPDH enzyme (YG-1 gene) in 9, 18, or 27 tandem repeats of the gene separated by clostripain cleavage sites (Park et al., 1998). Contrary to expectations, the highest expression level of YG-1 was observed for the 9-mer (67% of total proteins), a fact related to the use of the T7 promoter (Park et al., 1998).

Fida et al. (2009) first reported the direct insertion of a small tandem peptide multimer 'gene' into an expression vector without a fusion protein tag. In this example, the ACEIp 'fragmented peptide B' (PTHIKWGD), retrieved from thermal hydrolysates of tuna meat, was produced in *E. coli* as 6-mer. Although bypassing digestion steps to remove the fusion tag, this approach required a prohibitively expensive anti-peptide antibody affinity chromatography. Recently, the expression levels of this peptide B in *E. coli* have been improved by multimerization of the tandem sequence. The original 4-mer peptide B 'genes' were further assembled as 1, 2, 4 and 8x tandem repeats and fused to a his-tag. A correlation between the degree of multimerization and the expression level of these multimer 'genes' was observed, with the 32 multimer (8 x 4mer) presenting the highest expression (45.2% of total protein). The purified peptide monomers presented antihypertensive activity in SHR, decreasing systolic BP by 36.5 mm Hg upon 4 h of oral administration (Li et al., 2015).

Another example is the expression of a sequence encoding IYPR, an ACEIp isolated from sake and sake lees, as a 7-copy tandem repeat, linked by trypsin cleavage sites. Following cleavage with trypsin and purification by affinity chromatography, the resultant ACEIp presented antihypertensive activity in 10-week old SHR, significantly reducing systolic BP by 50 mm Hg, upon 4h of a single oral administration (Huang et al., 2012). Similarly, the DNA-coding sequence for GVYPHK, derived from a partially purified autolysate of bonito bowels, was linked by a trypsin cleavage site to form a 10-mer tandem protein (Wang et al., 2015). A single oral administration to SHR of the ACEIp obtained by trypsin cleavage, significantly reduced systolic BP already after 2 h of ingestion.

ACEI peptides fused to proteins/polypeptides

In a different approach, single or tandem ACEIp can be fused to proteins and expressed as high molecular weight fusion proteins. The use of fusion protein partners has several advantages: boosts peptide expression by increasing mRNA translation; helps short polypeptides stabilization; and facilitates the purification of the peptides by affinity chromatography. An additional proteolytic step is usually required after translation to release the ACEIp from the fusion partner. The enzyme glutathione S-transferase (GST) has been the most commonly used fusion partner, but ubiquitin, dihydrofolate reductase (DHFR) and maltose-binding protein (MBP) have also been used.

The GST fusion protein system has been used both in single and tandem ACEIp production. A 6-mer tandem of KVLPVP, derived from the milk β-casein hydrolysate, was linked by clostripain cleavage sites and fused to GST (Liu et al., 2007). The pure peptide presented an IC_{50} of 4.6 μ M and showed antihypertensive activity in SHR, dramatically decreasing systolic BP in a dose-dependent manner. Another example comprises recombinant concatemers of multiple IYVKY copies, fused to GST. Tandems of 2, 4 and 6-mer of the IYVKY sequence, linked by the chymotrypsin cleavage site and fused N-terminally to a His-tag and C-terminally to GST, were produced. Their expression in *E. coli* BL21 lead to a good yield production of bioactive single ACEIp IY and VKY (Oh et al., 2002).

The GST expression system was also used to produce single peptides, such as the ACEI BP1-3, derived from bovine β-casein (Losacco et al., 2007), and their precursors (Pro-BP1-3) (Losacco et al., 2007). Each Pro-BP or BP-coding DNA sequences were cloned downstream the GST sequence and flanked in both sides by the cleavage sites (3-5 aa long) of a membrane proteinase from *Lactobacillus helveticus* PR4. The use of this membrane proteinase was strategic, given the common use of this LAB in manufacturing dairy products that naturally contain similar ACEIp. However, the IC50 of BP1-3 was relatively high (Table 2).

Besides GST, other fusion proteins have been used in recombinant ACEIp production. BP1-3 and ProBP1-3 were also expressed in a probiotic strain of *Bifidobacterium pseudocatenulatum* , fused at the 5' end to a Shine-Dalgarno ribosome binding site (RBS) consensus sequence (Losurdo et al., 2013). Although the ACE-inhibitory activity increased in cell-free extracts of all recombinant hosts, the expression of BP1 and BP3 and their precursor forms was not detectable by RP-HPLC. This was attributed to their hydrolysis by intracellular endopeptidases, such as aminopeptidases and iminopeptidases. Other example is the production of a single ACEIp from α_{s1}-casein FFVAPFPEVFGK (known as CEI_{12}), fused to DHFR. Nevertheless, relatively low yields (0.5 $mg.L^{-1}$) of the CEI_{12}-DHFR fusion protein were obtained upon IPTG induction in *E. coli* (Lv et al., 2003).

These last reports demonstrate the limitations of single ACEIp expression approaches, which may be partially overcome by using tandem ACEIp. The synthetic gene coding for the ACEIp HHL, derived from a Korean soybean paste, was tandemly multimerized to a 40-mer and ligated to ubiquitin as a fusion gene (UH40). HHL monomers were recovered at 6.2 mg. L^{-1} yield (Jeong et al., 2007), showing the advantages of fusing proteins with ACEI tandem multimers, in comparison to single peptide approaches. Sixteen tandem repeats of the α-lactalbumin-derived ACEIp IW were N-terminally fused to MBP, and expressed in *E. coli* BL21 (Michelke et al., 2018). This MBP-IW fusion protein was recovered at low yield (0.52 mg soluble protein/g of*E. coli*) and, after hydrolysis with α-chymotrypsin, only 50.78 μg of IW monomers were released. Still,

the ACEI activity of the recombinant IW was indistinguishable from that of the chemically synthesized dipeptides.

Despite the moderate success of fusion protein approaches in *E. coli* , most require expensive and time-consuming purification steps to remove protein tags and the use of non-food-grade inducer molecules, such as IPTG. A recent study, based on the initial CEI_{12}expression in *E. coli* (Lv et al., 2003) and *Streptococcus thermophilus* (Renye and Somkuti, 2008; Renye and Somkuti, 2015) attempted to surpass these shortcomings by employing nisin-induced CEI_{12} expression in three LAB strains: *S. thermophilus* ST128, *Lactococcus lactis* subsp. *lactis* ML3, and *L. casei* C2. A synthetic CEI_{12}-coding gene was cloned under the nisA promoter, in-frame with the pediocin leader peptide to direct the secretion of the resultant fusion peptide by LAB hosts. Both *L. lactis* ML3 and *L. casei* C2 secreted the recombinant peptide, as confirmed by SDS-PAGE (Renye and Somkuti, 2015). Although recovered recombinant peptide yields were low, this study is a landmark, as it first reported the use of GRAS LAB species and of nisin as "food-grade" inducer, prospecting the use of LAB as ACEIP production platforms for the functional foods industry.

Multivariate ACEI peptides in bioactive polypeptides

A third strategy for bacterial expression of bioactive ACEIp relies on engineering synthetic genes with different ACEIp in tandem. Rao et al. (2009) described the design and production of a tandem antihypertensive peptide multimer (AHPM), as a precursor of 11 different ACEIp, joined by 1-3 aa-long linkers corresponding to cleavage sites of gastrointestinal proteases. The recombinant AHPM polypeptide, fused to a GST tag, was expressed in *E. coli* mostly as inclusion bodies, and reached a maximal production of 35% of total intracellular protein. Although the yield of multimer peptide recovery was low, simulated gastrointestinal digestion confirmed the release of highly active fragments from AHPM (Rao et al., 2009). This research team has also reported the design of a new polypeptide (BPP-1) composed of several ACEI and antioxidant peptides, tandemly linked by gastrointestinal proteases cleavage sites. The BPP-1 precursor consisted of a tandem multimer of the ACEIp: MRW, WIR, IRA, AMK, MKR, RGY, VAW, DGL, IPP, IKP, IKPFR, IKPVA, AKF, IW, VAF, VSV, IQY and IVY, and the antioxidant peptides DTHK, YPIL, FLEPDY, YLEPFR, YLEPDY, YDEPEW, HYRPFW, YEPDY and IWAPFY. To improve the yield of soluble form, BPP-1 was further fused to the tag 'cationic elastin-like polypeptide and SUMO' (cELP-SUMO). This tag both enhances the solubility of fusion proteins and allows its cleavage to efficiently release peptides (Rao et al., 2016). As a result, cELP-SUMO-BPP-1 was highly expressed in a soluble form in *E. coli* , consisting of approximately 52% of the total soluble proteins, with more than 70% of the fusion protein being expressed in a soluble form. After simulated gastrointestinal digestion of the purified BPP-1 the resulting hydrolysates exhibited notable *in vitro* ACE inhibitory and antioxidant activities (Rao et al., 2016).

Modified plant storage proteins containing ACEI peptides

The feasibility of modifying subunits of plant storage proteins to contain ACEIp has been widely tested in *E. coli* as primary evaluation step, envisaging their application in food crops. Two plant storage proteins have been used for ACEIp production: the soybean β-conglycinin α' subunit and the Amaranthus amarantin acidic subunit.

β-conglycinin α' subunit (Soybean). Matoba et al. (2001) introduced Novokinin (the RPLKPW ACEIp, a potent analogue of ovokinin) into three homologous sites of the soybean β-conglycinin α' subunit, by site-directed mutagenesis. This modified RPLKPW-containing α' subunit was first expressed in *E. coli* and recovered in a soluble fraction at yields of 15% of total protein (Matoba et al., 2001a). When orally administered, the undigested RPLKPW-containing α' subunit demonstrated a potent antihypertensive effect and a time-course behavior similar to the one of free RPLKPW peptide, denoting the rapid release of the peptide from the protein after ingestion. This study first attested the *in vivo* functionality of a modified storage protein containing ACEIp. Nevertheless, the release of the RPLKPW sequences from the modified subunit by gastrointestinal digestion was only about 30% in SHR. To overcome this limitation, Onishi and colleagues (Onishi et al., 2004) optimized the aa residues surrounding the three RPLKPW peptide units,

to facilitate *in vivo* release. Furthermore, a fourth RPLKPW sequence was also introduced to improve RPLKPW peptides yield. This new modified RPLKPW-containing α' subunit was efficiently expressed in *E. coli* as ˜25% of total bacterial proteins (a 10% yield increased relative to previously reported). Further, it significantly lowered systolic BP in SHR 4h after oral administration of a 2.5 $mg.kg^{-1}$ dose, one-fourth of the previously reported dose, proving the benefits of inserting a supplementary RPLKPW unit. Finally, a more potent antihypertensive protein was produced in *E. coli* , as an extension domain corresponding to residues 1-143 of the modified α' subunit, containing four RPLKPW sequences, with 1.0 $mg.kg^{-1}$ as the minimum effective dose.

Acidic subunit of amarantin (Amaranthus). Another plant storage protein that has been modified to produce active ACEIp is amarantin, the main seed storage protein of *Amaranthus hypochondriacus* . This modified amarantin acidic subunit has been extensively evaluated in *E. coli* , using different site directed mutagenesis strategies. Luna-Suárez and colleagues (Luna-Suárez et al., 2010) first reported the insertion of 4 tandem repeats of the ACEIp VY into the third hypervariable region of the amarantin acidic subunit, and named this chimeric protein 'bioamarantin'. The same group improved bioamarantin design by further inserting one copy of RIPP (Castro-Martínez et al., 2012) or IPP (Medina-Godoy et al., 2013) into the forth hypervariable region of the amarantin acidic subunit. All modified subunits showed higher ACEI activities than the native protein. Furthermore, the enzymatic hydrolysates of AMC3-containing ACEIp sequences (4xVY and IPP) presented a significant antihypertensive action at 100 $mg.kg^{-1}$ dose, 4.5h after oral administration in SHR (Medina-Godoy et al., 2013). Finally, this team modified the acidic subunit of amarantin by inserting 4 VY peptides into the fourth hypervariable region of the acidic subunit, ('AACM.4') (Morales-Camacho et al., 2016). AACM.4 showed highest expression levels, greater *in vivo* or kinetic stability and higher percentage of soluble form (˜5% of total protein), compared to the native protein or previously reported variants (Luna-Suárez et al., 2010; Castro-Martínez et al., 2012). Moreover, AACM.4 was also the most thermostable protein, suggesting that the fourth hypervariable region modification improves the subunit thermal stability. These results confirmed AACM.4 as a potential target for future plant transformation and food additive production. Importantly, the improvement of thermal stability can be a critical factor during functional food processing procedures.

Recombinant ACEI expression in plants and microalgae

Plant proteins are precursors of numerous ACEIp that can be released during gastrointestinal digestion or plant crops processing. However, given their low content in natural ACEIp, plant/plant-derived food consumption is usually insufficient to significantly lower BP. Furthermore, the industrial production of ACEIp, through enzymatic hydrolysis of plant proteins, can be troublesome and economically unviable due to high process costs and low ACEIp yield. The use of plant biotechnology (Figure 2 and Table 3) can thus expand the ACEI properties of plant crops, envisaging the establishment of novel plant-derived functional foods and food supplements.

Until now, rice (*Oryza sativa*) and soybean were the favored plant expression hosts for producing ACEIp. Some advantages include the existence of well-established transformation systems, high level of recombinant proteins production and high grain yield. Furthermore, the possibility of ACEIp' accumulation in rice and soybean seeds is highly beneficial for peptide stability and storage, providing a direct peptide delivery route (Twyman et al., 2003). Other reported systems of ACEIp production include cell suspension cultures and transplastomic platforms based on *C. reinhardtii* .

Figure 2 summarizes the main strategies for ACEIp production in plants and algae, and Table 3 provides detailed information. In Table 3 three main approaches are presented by chronological order of report: 1) the modification of storage proteins to carry ACEIp; 2) the generation of chimeras containing tandem repeats of ACEIp; 3) the generation of bioactive polypeptides containing multivariate ACEIp (Kim et al., 2008; Rosales-Mendoza et al., 2013).

Modified plant storage proteins containing ACEI peptides

Expression of ACEIp in plant storage proteins has the advantage of ensuring long-term protein stability and storage. Storage proteins are generally located in specialized compartments, such as protein bodies and vacuoles, which provide appropriate biochemical environments for protein/peptide accumulation, protecting those from proteolytic degradation (Twyman et al., 2003). ACEIp-coding sequences are generally introduced into homologous sites of the protein's variable regions to minimize changes in protein folding, productivity and localization in plants. Examples are the modified subunits of glutelin, β-conglycinin and amarantin.

Glutelin. Modified subunits of the rice storage protein glutelin, containing the potent ACEIp novokinin (RPLKPW), have been expressed in transgenic rice (Yang et al., 2006). Fusion proteins were expressed under the control of endosperm-specific glutelin promoters and specifically accumulated in seeds. Oral administration in SHR of either the RPLKPW-glutelin fraction or the transgenic rice seeds significantly reduced systolic BP, confirming the potential as valid nutraceutical delivery systems for antihypertensive peptides.

β-conglycinin α' subunit (Soybean). Based on the findings above described for *E. coli* (Matoba et al., 2001a; Onishi et al., 2004), a modified β-conglycinin α' subunit carrying 4 novokinin peptide units has been expressed in soybean (Nishizawa et al., 2008). However, the chimeric protein was only 0.2% of the total extracted protein from the transgenic soybean seeds, a low value to assess the seeds' *in vivo* effects. More recently, novokinin was expressed in transgenic soybean seeds as a 4 tandem multimer of novokinin fused to the β-conglycinin α' subunit. The chimeric protein produced in transgenic soybean seeds comprised 0.5 % of total soluble protein and 5 % of total β-conglycinin α' subunit. This chimeric protein was shown to possess antihypertensive activity, reducing systolic BP in SHR after 0.15 $g.kg^{-1}$ protein extract oral administration. A similar effect was attained following administration of 0.25 $g.kg^{-1}$ dose of defatted flour (Yamada et al., 2008).

Acidic subunit of amarantin (Amaranthus). Based on previous studies in *E. coli* (Luna-Suárez et al., 2010), bioamarantin was also expressed in cell suspension cultures of*Nicotiana tabacum L.* NT1. Protein hydrolysates of transgenic*calli* showed high ACEI activity, with 3.5 $\mu g.ml^{-1}$ IC50 value , 10-fold lower than protein extracts of wild-type cells (IC50 of 29.0 $\mu g.ml^{-1}$) (Santos-Ballardo et al., 2013). This was the first report of the production of a chimeric protein comprising ACEIp in plant cell suspension cultures. More recently, bioamarantin was also expressed in transgenic tomato fruits, and stably accumulated at levels up to 12.71% of total protein content. Furthermore, a remarkable increase (5–22 %) in total protein content was also observed in transgenic tomato fruits, when compared to non-transformed ones. Protein hydrolysates from transgenic tomato fruits showed 0.376 to 3.241 $\mu g.ml^{-1}$*in vitro* IC50 values, corresponding to an increase of up to 13-fold in ACEI activity, when compared to the non-transformed fruits (Germán-Báez et al., 2014). Positive reports of the expression of modified amarantin variants in *E. coli* (Luna-Suárez et al., 2010; Medina-Godoy et al., 2013; Morales-Camacho et al., 2016) along with their sustained expression in tomato (Germán-Báez et al., 2014) and tobacco (Santos-Ballardo et al., 2013), prospect the high scale production of ACEIp-containing modified amarantin in heterologous hosts.

Tandem repeats of ACEI peptides

Following the first attempts of novokinin production in modified storage proteins in soybean (Nishizawa et al., 2008; Yamada et al., 2008) and rice (Yang et al., 2006); Wakasa et al. (2011) aimed to generate transgenic rice seeds that would accumulate higher amounts of novokinin. Their strategy comprised the expression of 10 or 18 tandemly repeated novokinin sequences with a KDEL endoplasmic reticulum-retention signal at the C-terminus, using the glutelin promoter and its signal peptide. Although the chimeric protein was unexpectedly accumulating in the nucleolus and at a low level, significant antihypertensive activity was detected after a single oral dose in SHR. More importantly, this effect was observed over 5-wk at doses as low as 0.0625 $g.kg^{-1}$.

More recently, a synthetic gene containing tandem repeats of the VLPVP ACEIp has been expressed in

a transplastomic *C. reinhardtii*strain. VLPVP-coding sequences were linked by cleavage sites of pepsin, trypsin, and chymotrypsin. Biomass of recombinant *C. reinhardtii*was *in vitro* digested, and the VLVPV peptide was identified and quantified by HPLC. The highest expression line produced 0.292 mg recombinant protein/mg freeze-dried biomass. Intragastric administration to SHR, at a dose of 30 $mg.kg^{-1}$, significantly reduced rats systolic BP (Ochoa-Mendez et al., 2016). This *in vivo*antihypertensive effect first provided a functional validation of using ACEIp-producing microalgae as food supplements for hypertension patients.

Multivariate ACEI peptides in bioactive polypeptides

Campos-Quevedo et al. (2013) reported the design and production of a milk-derived chimeric protein containing sequences for multifunctional bioactive peptides, including peptides with hypocholesterolemic, antihypertensive, opioid, and antimicrobial activities. The precursor chimeric protein contained 20 different bioactive peptides linked by gastrointestinal protease cleavage sites. The synthetic gene coding for the milk-derived chimeric protein was transferred to *C. reinhardtii* using biolistic bombardment. Transplastomic transformants containing the target synthetic gene were identified, and ELISA quantification assay revealed that the expressed chimeric protein accumulated at levels ranging between 0.16 and 2.4% of total soluble protein. Although no functional studies (*in vitro* ACEI activity or *in vivo* antihypertensive effect) were performed, this study first showed the potential of *C. reinhardtii* as expression platform for the production of ACEIp in multifunctional bioactive polypeptides.

The future of ACEI production

ACEI are a major first line treatment in managing hypertension, but synthetic ACEI carry side-effects.

Food-derived ACEIp have been effective in BP reduction without adverse effects and can be used as an alternative to synthetic ACEI. Potent ACEIp have been isolated from whole-food protein hydrolysates of both animal and plant origin. However, protein hydrolysis-based methods generate complex peptidic mixtures from which ACEIp must be purified, increasing production costs. Besides, the resulting recovery yields and ACEIp bioavailability are low, rendering the process economically unviable.

The use of recombinant technology for the production of ACEIp can extend its application for functional foods and pharmaceutical purposes as it allows the large-scale and low-cost production. *E. coli* has been the preferred host for recombinant ACEIp production, but lactic acid bacteria (LAB), plants (predominantly cereals and legumes) and the microalgae *C. reinhardtii* have also emerged as ACEIp production hosts.

Bacteria, such as *E. coli* have the advantage of growing fast and not requiring complex culture systems. A diverse set of molecular tools available for ACEIp production in *E. coli* including, a wide range of vectors, promotors and inducers, provide also an important competitive advantage. Furthermore, LAB species with its GRAS status and extensive use in the dairy industry are important ACEIp production hosts for the functional foods industry.

Nevertheless, in recent years, the potential use of plant biotechnology for the large-scale production of pharmaceutically relevant proteins and peptides has significantly increased. Given their low-price and safety, plants offer many advantages for producing valuable recombinant proteins and peptides, as ACEIp, when compared to mammalian cell cultures (Gomes et al., 2019). Further, plants are very versatile, encompassing a wide range of production platforms, from transgenic plants to cell suspension cultures. The use of rice and soybean, for example, allows ACEIp accumulation in edible seeds, a direct delivery vehicle with improved protein stability and storage (Twyman et al., 2003), with soybeans seeds accumulating up to 40% protein (dry weight). Legumes as soybeans produce more proteins than other plants, being promising host systems for molecular farming. Alternatively, tomato fruits are palatable as raw tissue and can be lyophilized and stored for a long time (Lico et al., 2012). The possibility of using plant tissues as direct oral delivery means, is a major differentiating factor of bioactive peptide production in plant platforms, compared to mainstream production platforms, such as*E. coli* . In bacteria, the produced peptides must undergo complex purification procedures,

and processed into a consumable product. Plant cell suspension cultures are alternatives to transgenic plants; tobacco*calli* cultures proliferate rapidly, and technologies for gene transfer and expression are well-established for this species (Pires et al., 2012; Schillberg et al., 2013). Further, plant cell suspension cultures grown in sterilized contained environments provide a cGMP-compatible production environment, advantageous to the pharmaceutical industry and regulatory authorities (Paul et al., 2013; Spök et al., 2008). Chloroplast-based expression platforms also attract great interest in mass scale production of ACEIp, given its high recombinant protein expression levels (Fletcher et al., 2007). The algae model *C. reinhardtii* has fast growth rates, GRAS designation, and can be grown in contained environments (González-Ortega et al., 2015). Direct oral delivery of ACEIp in this host is also an option, as green algae are edible and do not contain endotoxins, human viral or prion contaminants (Mayfield et al., 2007). All these expression platforms are compatible with the above described genetic engineering strategies to improve ACEIp expression, including the multimerization of small peptides in tandem repeats, their fusion with (or insertion in) highly expressed proteins - with the plus of targeting/ accumulating the ACEIp-carrying chimeric proteins in seeds - and the use of similar chimeras, assembling different ACEI and other peptides into multifunctional bioactive polypeptides/proteins. Together with the use of gastrointestinal proteases cleavage sites to flank the peptides, these approaches are perfect for the adequate release of pharmacologically relevant peptides in edible platforms, for preventive or therapeutic purposes.

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Authors' declaration

The authors have no conflict of interest to declare.

Figure captions

Figure 1 . The renin-angiotensin and the kallikrein-kinin systems in the regulation of blood pressure. The intricate interplay between these two systems, dependent on the ACE, is the basis of the control of blood pressure. While the renin-angiotensin mechanism leads to vasoconstriction and water retention, increasing blood pressure, the kallikrein-kinin system has opposite effects by promoting vasodilation.

Figure 2. Overview of cloning and expression of ACEIp-coding synthetic genes in plants and algae. Three main cloning strategies are used: 1) tandem repeats of a target ACEIp sequence; 2) introduction of the ACEIp in the hypervariable region of storage proteins, and the 3) assembly of ACEI polypeptides with others bioactive peptides. These strategies can be applied in different plant/algae expression platforms, such as whole transgenic plants/algae, transplastomic plants/algae and plant/algae cell suspension cultures. The resulting ACEIp-enriched plants/algae can be used for direct human consumption (direct oral delivery) or processed by hydrolysis and purified to be used as food supplements. Images used are from Adobe Stock.

Tables

Table 1. Commercially available functional foods or food ingredients containing ACE inhibitory peptides.

Peptides	Food	Product (name)
FFVAPFPEVFGK	Milk-derived ingredient (casein-derived dodecapeptide)	C12 Peption, Casein DP Peptio Drink

Peptides	Food	Product (name)
LKPNM	Bonito-derived capsules; Bonito-derived tablets; Bonito-derived powder; Bonito-derived ingredients	Peptide ACE 3000, PeptACE; Vasotensin®; Peptide Tea; Levenorm®
AKYSY	Seaweed (*Porphyra yezoensis*) powder; Seaweed (*P. yezoensis*) beverage	Mainichi Kaisai Nori; Peptide Nori S
IPP and VPP	Sour milk tablet, beverage; Fermented milk enriched in calcium	Ameal S, Ameal S 120; Evolus®
LVY	Sesame-derived beverage	Goma Pepucha
VY	Sardine-derived ingredient; Sardine-derived beverage; Sardine-derived tablet	Valtyron®; Lapis Support Sato Marine Super P
VY, IY and FY	Seaweed (*Undaria pinnatifida*) jelly	Wakame Jelly
VY, IY and IVY	Royal jelly beverage	StayBalance RJ
Bonito peptides	Bonito-derived soup	Peptide Soup
Whey peptides	b-LG hydrolysate	BioZate
Mushroom peptides	Mushroom-derived powder	Bunaharitake

Table 2. ACEI peptides expressed as recombinant chimeric constructs in bacterial platforms. *Escherichia coli* strains BL21(DE3), JM109, HMS174(DE3), Origami (DE3) were the mainly used hosts. Other hosts include LAB species such as *Lactococcus lactis* ,*Lactobacillus casei* (Renye and Somkuti, 2015) and the probiotic*Bifidobacterium pseudocatenulatum* (Losurdo et al., 2013). '[peptide]': yield in amount of purified peptide (mg per L of culture); 'ACEI IC50': ACE inhibitory in vitro activity (IC50, in µM); 'Anti-HT': antihypertensive activity (mmHg); 'Dose': dose of peptides in mg per Kg of body weight.

Peptide(s)	Construct strategy	[peptide] ($mg.L^{-1}$)[b]	ACEI IC_{50} (µM)	Anti-HT (mmHg)	Dose ($mg.kg^{-1}$)[c]	Ref.
YG-1 (GHKIATFQER)	**Tandem repeats** 9, 18 or 27x YG-1 linked by the cleavage site of clostripain;	105 (5h)	= natural YG-1	ND	ND	(Park et al., 1998)
KVLPVP	**Tandem repeats** 6x KVLPVP linked by the cleavage site of clostripain; GST tag	170 (5h)	4.6	-21.4 (4h)	0.3	(Liu et al., 2007)
Tuna AI (PTHIKWGD)	**Tandem repeats** 6x Tuna AI	105–115 (8h)	2.0	ND	ND	(Fida et al., 2009)

Peptide(s)	Construct strategy	[peptide] ($mg.L^{-1}$)[b]	ACEI IC_{50} (μM)	Anti-HT (mmHg)	Dose ($mg.kg^{-1}$)[c]	Ref.
	Tandem repeats 4, 8, 16 or 32x Tuna AI; N and C-terminal His tag	218.9 (5h)	1.2	-36.5 (4h)	2.0	(Li et al., 2015)
IYPR	**Tandem repeats** 7xIYPR linked by the cleavage site of trypsin; N-terminal enterokinase cleavage site; N-terminal His tag	1.8 (7h)[1]	111.3	-50 (4h)	0.4	(Huang et al., 2012)
GVYPHK	**Tandem repeats** 10x GVYPHK linked by the cleavage site of trypsin; N-terminal His tag	13.8 (5h)	ND	-42.6 (2h)	0.8	(Wang et al., 2015)
HHL	**Tandem + Fusion with Ubiquitin** 40xHHL; C-terminal His tag	6.2 (4h)	8.4	ND	ND	(Jeong et al., 2007)
CEI_{12} (FFVAPFPEVFGK)	**Fusion with DHFR** 1xCEI_{12}	0.5 (5h)[2]	ND	ND	ND	(Lv et al., 2003)
	Fusion with pediocin leader peptide 1xCEI_{12}	ND	ND	ND	ND	(Renye and Somkuti, 2015)
IW	**Tandem + Fusion with MBP** 16xIW; N-terminal MBP affinity tag	0.51 (7h)[1]	1.72[3] 5.15[4] 11.08[5]	ND	ND	(Michelke et al., 2018)

Peptide(s)	Construct strategy	[peptide] ($mg.L^{-1}$)[b]	ACEI IC_{50} (μM)	Anti-HT (mmHg)	Dose ($mg.kg^{-1}$)[c]	Ref.
IY and VKY	**Tandem + Fusion with GST** 2, 4 or 6x IYVKY linked by the cleavage site of chymotrypsin; N-terminal His tag	38 (4h)[6]	18.53[6]	ND	ND	(Oh et al., 2002)
BP1 (SLVYPF-PGPI) BP2 (NIP-PLTQTPV) BP3 (DKIHPF)[7]	**Single peptides + Fusion with GST** 1xBP1, 1xBP2 or 1xBP3 flanked by the cleavage sites of a proteinase from *L. helveticus* PR4	1.5 (6h)	BP1 467.03 BP2 807.18 BP3 1729.21	ND	ND	(Losacco et al., 2007)
BP1, BP2 and BP3 Pro-BP1, Pro-BP2, Pro-BP3	**Single peptides + Fusion with RBS** 1xBP1, 1xBP2 or 1xBP3 and their precursor forms, fused to Shine-Dalgarno RBS sequence for *B. pseudo-catenulatum* at the 5' end	NR	ND (increased in extracts)	ND	ND	(Losurdo et al., 2013)
VWIS, VW, RIY, IY, LW, IKW, LKPNM, LKP, RPLKPW, NMAINPSK, IPP	**Multimer of ACEIPs** tandem multimer of 11 ACEIP linked by cleavage sites of gas-trointestinal proteases; GST tag	399 (5h)[2]	33[2]	ND	ND	(Rao et al., 2009)

Peptide(s)	Construct strategy	[peptide] ($mg.L^{-1}$)[b]	ACEI IC_{50} (μM)	Anti-HT (mmHg)	Dose ($mg.kg^{-1}$)[c]	Ref.
MRW, WIR, IRA, AMK, MKR, RGY, VAW, DGL, IPP, IKP, IKPFR, IW, IKPVA, AKF, IQY VAF, VSV, IVY	**Multimer of bioactive peptides** tandem multimer of 18 ACEIP and 9 antioxidant peptides linked by cleavage sites of gastrointestinal proteases; N-terminal cELP-SUMO tag	167.2 (20h)[2]	0.28[2]	ND	ND	(Rao et al., 2016)
Novokinin (RPLKPW)	**Μοδιφιεδ β-ϛονγλψϛινιν α′ συβυνιτ** 1x Novokinin in 3 homologous sites of the core domain flanked by trypsin and/or chymotrypsin cleavage sites	5.2 (42h)[2]	ND	-25 (4h)[2]	10[2]	(Matoba et al., 2001a)
	Μοδιφιεδ β-ϛονγλψϛινιν α′ συβυνιτ 1x Novokinin in 3 homologous sites of the core domain plus 1x Novokinin in a homologous site of the extension domain; flanked by gastrointestinal proteases cleavage sites	ND	ND	-18 (4h)[2]	2.5[2]	(Onishi et al., 2004)

Peptide(s)	Construct strategy	[peptide] ($mg.L^{-1}$)[b]	ACEI IC_{50} (μM)	Anti-HT (mmHg)	Dose ($mg.kg^{-1}$)[c]	Ref.
VY	**Modified amarantin acidic subunit** 4xVY in the third variable region (VR3); C-terminal His tag **AACM.3**	60 (3h)[2]	2.0[2]	ND	ND	(Luna-Suárez et al., 2010)
	Modified amarantin acidic subunit 4xVY in the fourth variable region (VR4); C-terminal His tag **AACM.4**	560 (6h)[2]	ND	ND	ND	(Morales-Camacho et al., 2016)
VY and RIPP	**Modified amarantin acidic subunit** 4xVY in VR3 and 1xRIPP in VR4; C-terminal His tag **AACM3.4**	50 (3h)[2]	1.41[2]	ND	ND	(Castro-Martínez et al., 2012)
VY and IPP	**Modified amarantin acidic subunit** 4xVY in VR3 and 1xIPP in VR4; C-terminal His tag **AMC3**	ND	5640[2]	-58 (4.5h)[2]	100[2]	(Medina-Godoy et al., 2013)

[a]T7 promoter was used in all reports, except in (Lv et al., 2003) (T5 promoter), (Liu et al., 2007) and (Rao et al., 2009) (tac promoter) and (Renye and Somkuti, 2015) (nisA promoter)

[b]The presented values correspond to the higher peptide expression levels obtained. Peptide expression was induced with IPTG, excepting (Renye and Somkuti, 2015), where nisin was used instead.

[c]Peptides were orally administered, excepting (Medina-Godoy et al., 2013), where recombinant protein hydrolysates were administered by gastric gavage.

ND- Not determined

NR- Not recovered

[1]mg of peptide per g of wet weight cells

[2] Value for the chimeric protein

[3] Value for Rabbit lung ACE

[4] Value for Human plasma ACE

[5] Value for Human umbilical vein endothelial cells (HUVECs) ACE

[6]Value for the peptide multimer

[7]BP1-3 complete amino acid sequences (the bioactive sequences are underlined): BP1, QTQ*SLVYPFPGPI* PNS; BP2, LPQ*NIPPLTQTPV* VVP; and BP3, EDELQ*DKIHPF* AQTQS. Correspond to fragments 57–66, 73–82, and 47–52 of bovine β-casein (A^2 allelic variant), respectively.

Table 3. ACEI peptides expressed as recombinant constructs in plant and algae platforms. 'Anti-HT': antihypertensive activity (mmHg); 'Dose': dose of peptides in g per Kg of body weight. HVR, hypervariable region; VR, variable region.

System	Host (accumulation region)	ACEI peptide (s)	Construct strategy	Administration mode	Anti-HT (mmHg)	Dose ($g.kg^{-1}$)	Ref.
Whole transgenic plant	Rice (seeds)	Novokinin (RPLKPW)	**Fusion with glutelin B1 (pGluB1-HRP)** 2x Novokinin in 2 HVR of GluB1 acidic subunit plus 1x Novokinin in the C terminal VR of GluB1 basic subunit	oral administration of crude glutelin B1 fraction	-28±7 (4h)	0.03	(Yang et al., 2006)
				oral administration of unpolished pulverized GluB1-HRP rice seeds	Not significant	1.00	

System	Host (accumulation region)	ACEI peptide (s)	Construct strategy	Administration mode	Anti-HT (mmHg)	Dose ($g.kg^{-1}$)	Ref.
			Fusion with glutelin A2 and C (pGluA2-HRP/GluC-HRP) 2x Novokinin in 2 HVR of GluA2 and GluC acidic subunits	oral administration of unpolished pulverized GluA2-HRP/GluC-HRP rice seeds	-15.6±4.8 (2h)	1.00	
			Tandem structure 10x Novokinin + KDEL; 18x Novokinin + KDEL	oral administration of transgenic seeds containing 18x novokinin	-15.6±1.1 (4h)	1.00	(Wakasa et al., 2011)
	Soybean (seeds)	Novokinin (RPLKPW)	**Φυσιον ωιτη β-ϛονγλψϛινιν α′ συβυνιτ** 1x Novokinin in 4 sites	oral administration of β-conglycinin fraction	-16.0±1.5 (8h)	0.15	(Yamada et al., 2008)
				oral administration of defatted flour	-16.4±4.3 (6h)	0.25	
			Φυσιον ωιτη β-ϛονγλψϛινιν α′ συβυνιτ 4x Novokinin	ND	ND	ND	(Nishizawa et al., 2008)
	Tomato (fruits)	VY	**Fusion with acidic subunit of amarantin** 4x VY + KDEL	ND	ND	ND	(Germán-Báez et al., 2014)

System	Host (accumulation region)	ACEI peptide (s)	Construct strategy	Administration mode	Anti-HT (mmHg)	Dose ($g.kg^{-1}$)	Ref.
Cell suspension cultures	Tobacco (*calli)*	VY	**Fusion with acidic subunit of amarantin** 4x VY + KDEL	ND	ND	ND	(Santos-Ballardo et al., 2013)
Transplastomic	*C. reinhardtii* (chloroplasts)	VLPVP	**Tandem structure** 6x VLPVP	Oral administration of freeze-dried biomass	-20 (6h)	0.30	(Ochoa-Mendez et al., 2016)
		GLDIQK VAGTWY LDAQS-APLRLLQK VLVLDT-DYK CMENSA ALPMHIR	**Multivariate chimeric peptide**	ND	ND	ND	(Campos-Quevedo et al., 2013)

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